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Gibberellin-Regulated Expression of a *myb* Gene in Barley Aleurone Cells: Evidence for Myb Transactivation of a High-pI α-Amylase Gene Promoter

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Functional analysis of a barley high-pI α-amylase gene promoter has identified a gibberellin (GA) response complex in the region between −174 and −108. The sequence of the central element, TAACAAA, is very similar to the c-Myb and v-Myb consensus binding site. We investigated the possibility that a GA-regulated Myb transactivates α-amylase gene expression in barley aleurone cells. A cDNA clone, *GAmyb*, which encodes a novel Myb, was isolated from a barley aleurone cDNA library. RNA blot analysis revealed that *GAmyb* expression in isolated barley aleurone layers is up-regulated by GA. The kinetics of *GAmyb* expression indicates that it is an early event in GA-regulated gene expression and precedes α-amylase gene expression. Cycloheximide blocked α-amylase gene expression but failed to block *GAmyb* gene expression, indicating that protein synthesis is not required for *GAmyb* gene expression. Gel mobility shift experiments with recombinant GAMyb showed that GAMyb binds specifically to the TAACAAA box in vitro. We demonstrated in transient expression experiments that GAMyb activates transcription of a high-pI α-amylase promoter fused to a β-glucuronidase reporter gene in the absence of GA. Our results indicate that the GAMyb is the sole GA-regulated transcription factor required for transcriptional activation of the high-pI α-amylase promoter. We therefore postulate that GAMyb is a part of the GA-response pathway leading to α-amylase gene expression in aleurone cells.

INTRODUCTION

The barley aleurone layer provides a convenient system for the study of molecular mechanisms involved in gibberellin (GA)-regulated gene expression. Following the addition of GA, a rapid increase occurs in α-amylase gene expression in isolated barley aleurone layers, and this effect is inhibited by abscisic acid. Studies over many years are gradually identifying the steps involved between GA perception and α-amylase synthesis and secretion (reviewed in Jacobsen et al., 1995).

No GA receptor has yet been identified, but progress has been made toward localization of the site of GA perception in aleurone cells. Evidence from experiments using gibberellin A_4 (GA_4) covalently bound to Sepharose beads and anti-idiotype antibodies suggests that GA is perceived on the plasma membrane in oat aleurone protoplasts (Hooley et al., 1991, 1992; Smith et al., 1993). This is supported by more recent work showing that microinjection of GA_3 in barley aleurone protoplasts fails to induce α-amylase synthesis and secretion. However, when external GA_3 is applied, the protoplasts respond, indicating that the site of perception is on the external face of the plasma membrane (Gilroy and Jones, 1994).

Little is known about the molecular events that transmit the GA signal through the cytoplasm from the receptor and ultimately trigger expression of genes encoding α-amylase and other hydrolytic enzymes. It is not known whether the GA response in aleurone cells is mediated by a single pathway or whether different genes respond to different pathways. GA-induced changes in cytosolic calcium in the peripheral cytoplasm of aleurone protoplasts are thought to play a role in GA signal transduction, but as yet there is no direct evidence that these changes directly result in increases in gene expression (Bush and Jones, 1990; Gilroy and Jones 1992).

An important step toward understanding the molecular mechanisms of hormonal regulation of α-amylase gene expression is the identification of *trans*-acting regulatory proteins that interact with *cis*-acting elements within the GA response complex (GARC). Functional analysis of barley high-pI α-amylase promoters has identified a GARC consisting of the pyrimidine, TAACAAA, and TATCCAC boxes, which are necessary for the GA response (Skriver et al., 1991; Gubler and Jacobsen, 1992; Rogers et al., 1994; F. Gubler, T. Wallace, and J.V. Jacobsen, unpublished data). Analyses of a barley low-pI α-amylase promoter suggest that GA probably also acts through similar

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cis-acting elements, but additional *cis*-acting elements upstream of the pyrimidine box are also important for the full response (Lanahan et al., 1992; Rogers and Rogers, 1992).

Identification of *cis*-acting elements has led to the search for factors, presumably proteins, that interact with the sequences and regulate α-amylase gene expression. DNA sequences that bind nuclear proteins in vitro have been identified in cereal α-amylase promoters by using DNase I footprinting and gel mobility shift assays (Ou-Lee et al., 1988; Rushton et al., 1992; Sutliff et al., 1993; Goldman et al., 1994). Two recent studies have shown that GARC sequences in wheat and barley α-amylase promoters can act as binding sites for nuclear factors. Sutliff et al. (1993) characterized nuclear factors from barley aleurone layers that bind to sequences from a barley low-pI α-amylase gene. A GA-dependent binding factor was shown to bind specifically to sequences that coincide with the TAACAGA and TATCCAT boxes and proximal sequences. It is not yet clear whether this binding factor contains a single nuclear protein that binds to both elements or whether it consists of two or more proteins with different binding specificities. Rushton et al. (1992) demonstrated that nuclear factors from GA-treated oat protoplasts bind specifically to a number of sites, including the pyrimidine and TAACAGA elements in a low-pI wheat α-amylase promoter. It is not known whether the binding is GA inducible.

Consideration of the GARC *cis*-acting elements led us to focus on the possibility that they interact with Myb-like transcription factors. The c-Myb and v-Myb consensus binding sequence, YAACKGHH (where Y is C/T, K is G/T, and H is A/C/T; Weston, 1992), is similar to the sequence of the central GARC element, TAACAAA, in a high-pI α-amylase gene promoter (Jacobsen and Close, 1991; Skriver et al., 1991; Gubler and Jacobsen, 1992) and is also similar to the corresponding TAACAGA sequence in a barley low-pI α-amylase promoter (Lanahan et al., 1992; Rogers and Rogers, 1992). Both of these GARC elements contain the core AAC sequence of the Myb binding site (Tanikawa et al., 1993). There is experimental support for a role of Myb-like transcription factors in GA_3-regulated gene expression in barley aleurone. RNA blot analysis of *myb* gene expression in barley aleurone layers using the maize *C1 myb* gene as a probe detected three different *myb*-related mRNA size classes (Jacobsen and Gubler, 1993). Two of the *myb*-related mRNA classes were down-regulated by GA, and one class was up-regulated.

We report the isolation and characterization of a cDNA clone, *GAmyb*, encoding a Myb-related protein synthesized in barley aleurone layers. Studies of the expression pattern of the *GAmyb* gene in response to hormone treatments and the protein synthesis inhibitor cycloheximide indicated that *GAmyb* gene expression precedes α-amylase gene expression in GA_3-treated aleurone layers. We show that GAMyb binds to the central GARC element, the TAACAAA box, in a sequence-specific manner. The GAMyb protein could transactivate the α-amylase promoter in the absence of exogenously added GA, thus indicating that it is an integral part of the GA response pathway.

RESULTS

Putative Myb Binding Sites in the Promoter of a Barley High-pI α-Amylase Gene

The central element of the GARC, the TAACAAA box and 3′ adjoining sequences, contains two putative Myb binding sites (Figure 1), based on the c-Myb and v-Myb consensus binding site, YAACKGHH (Weston, 1992). The 5′ site, TAACAAAC, covers the whole TAACAAA box and closely matches the consensus c-Myb and v-Myb binding site (six of eight bases). The 3′ site, AAACTCGG, only partly covers the 3′ end of the TAACAAA box and shows less identity with the consensus binding site (five of eight bases). To test whether Myb-related proteins are involved in α-amylase gene expression by binding to the TAACAAA box and activating transcription, we cloned cDNAs that encode GA-regulated Myb-related proteins and tested their function.

Cloning of *GAmyb* cDNA and Sequence Comparison with Myb-Related Proteins

Myb proteins contain two similar 51 to 53 amino acid repeats (R2 and R3) that are important for DNA binding (Ogata et al., 1994). Taking advantage of the amino acid sequence conservation in the DNA binding domains of animal and plant Mybs, we screened a cDNA library prepared from hydrated barley aleurone layers with a 170-bp probe containing the sequence coding for the DNA binding domain (R2 and R3 repeats) of the maize C1 Myb protein (Paz-Ares et al., 1987). From an initial screen of 10^6 recombinant phages, one partial cDNA clone, *GAmyb*, was isolated that contained a 1991-bp insert. 5′ rapid amplification of cDNA ends (RACE) with nested primers was used to amplify sequences at the 5′ end of the gene transcript (Frohman, 1990). The longest polymerase chain reaction (PCR) product cloned was 408 bp, and sequence analysis showed that it extended the cDNA sequence an additional 271 bp, with 137 bp of exact overlap. Partial sequencing of a barley genomic clone containing the *GAmyb* gene confirmed the sequence of the 5′ RACE clone (data not shown).

The complete nucleotide sequence of the *GAmyb* cDNA, including the additional sequences of the 5′ RACE clone and the deduced amino acid sequence, is shown in Figure 2. The longest open reading frame extends 1659 bp from position 275

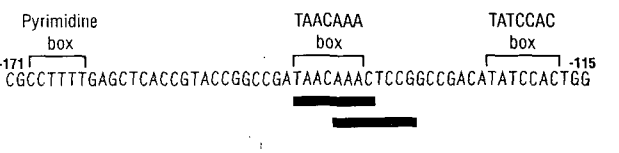

Figure 1. Potential Myb Binding Sites in the GARC.

The sequence of the high-pI α-amylase promoter in the region of the GARC. Potential Myb binding sites are underlined. Nucleotide numbers are relative to the transcription start.

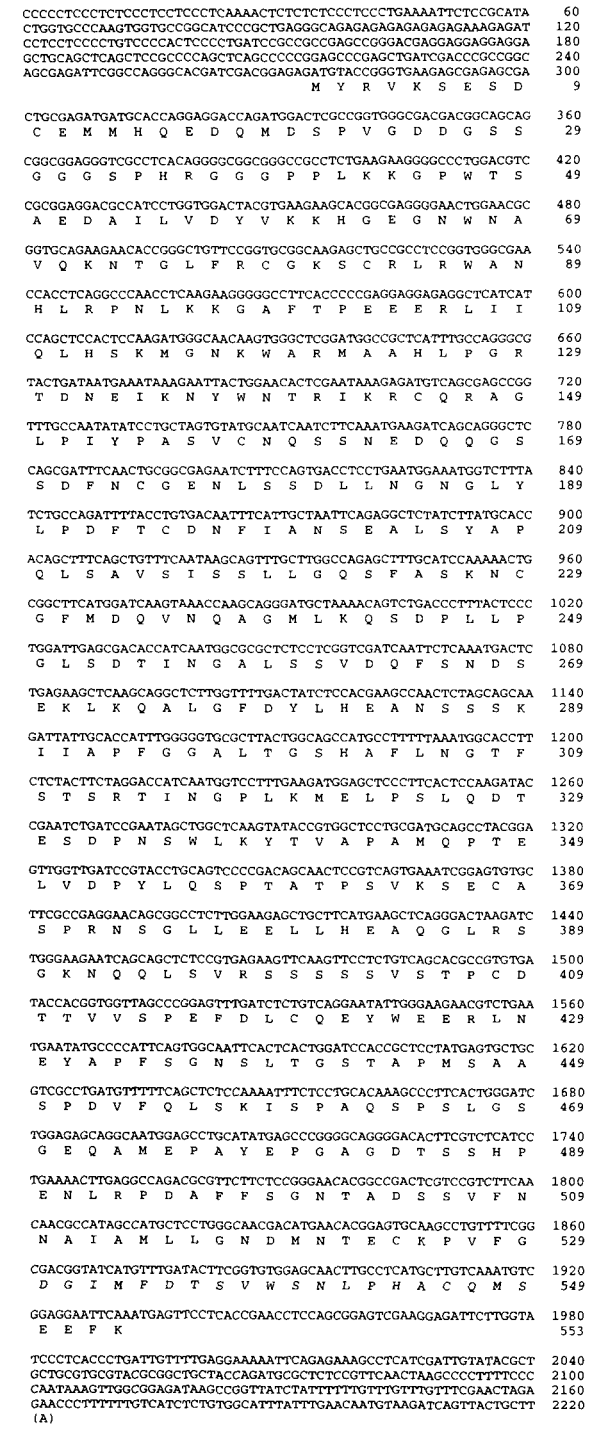

Figure 2. Nucleotide and Predicted Amino Acid Sequence of the *GAmyb* cDNA.

Nucleotides 1 to 271 correspond to the sequence obtained by 5′ RACE; nucleotides 272 to 2220 correspond to pGAmyb. Sequence data have been submitted to EMBL/GenBank/DDBJ as accession number X87690.

to a TGA stop codon found at position 1933 and encodes a polypeptide of 533 amino acid residues (molecular mass of 60 kD). The N-terminal portion of the protein contains a region that is highly conserved among Myb-related proteins. Figure 3 shows that residues 42 to 94 (R2) and 95 to 145 (R3) are highly homologous with the R2 and R3 regions of the DNA binding domain of animal and plant Myb proteins. Both of these repeats contain conserved tryptophans, which play a critical role in stabilizing the DNA binding domain of animal Mybs (Ogata et al., 1992). Sequence comparisons between R2 and R3 regions from other plant and animal Myb proteins show that the GAMyb is most closely related to MYB.Ph3 (86% identity), which is expressed in petunia flowers (Avila et al., 1993). There is less sequence identity between the R2 and R3 repeats of GAMyb and the other barley Myb proteins, HV1, HV5, and HV33 (54 to 63%) (Marocco et al., 1989; Wissenbach et al., 1993). Outside of the putative DNA binding domain, and nine amino acids immediately C terminus of the R3 region (146 to 154), there is a stretch of 15 amino acids in the C-terminal position of the GAMyb open reading frame (374 to 388) that shares 73% identity with amino acids 329 to 343 of MYB.Ph3. Interestingly, secondary structure prediction of both regions reveals that they both could form an amphiphathic α-helix, which is characteristic of activator domains (Paz-Ares et al., 1990; Avila et al., 1993).

DNA gel blot analysis of barley genomic DNA cut with HindIII and XbaI, using the 3′ end of the cDNA (from position 1430 to 2189) as a probe, showed only one band that hybridized with this probe (Figure 4). This result indicates there is only one copy of the *GAmyb* gene in the barley *myb* gene family. When the DNA was cut with BglII, two bands were observed with the 3′ probe. The appearance of two bands is due to an internal BglII site within the sequences that were probed.

GAmyb Gene Expression Is Regulated by GA

To test whether *GAmyb* gene expression was GA regulated, blots of RNA from control and GA_3-treated barley aleurone layers were probed using a 3′ gene-specific *GAmyb* cDNA probe. Figure 5A shows that *GAmyb* mRNA levels increased rapidly in response to GA_3. By 12 hr, the level in GA_3-treated aleurone layers was approximately fivefold higher than that found at the corresponding time for control treatments. However, the increase in mRNA levels was transient. Between 12 and 24 hr, the level of mRNA declined by 50%. In untreated aleurone layers, *GAmyb* mRNA levels remained low for the first 12 hr and then increased slightly over the next 12 hr. Accumulation of α-amylase mRNA levels in the same RNA samples is also shown, and the increase in response to GA_3 occurred later than seen for the *GAmyb* mRNA (Figure 5B).

To compare the kinetics of *GAmyb* and α-amylase mRNA accumulation in GA_3-treated barley aleurone layers, transcript levels in the blot shown in Figure 5A were quantified using a PhosphorImager and normalized to rRNA. The time course in Figure 5B shows that the increase in expression of the

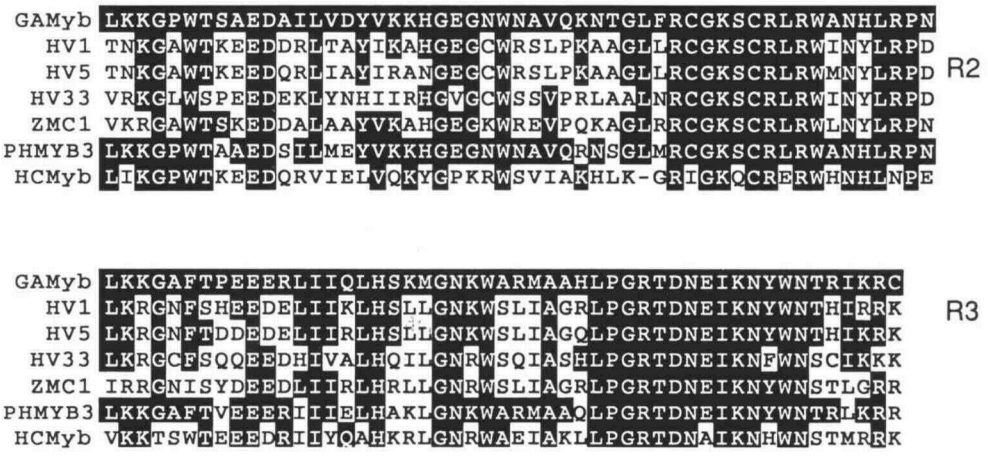

Figure 3. Comparison of Amino Acid Sequences of the Putative DNA Binding Domain of GAMyb and Other Myb-Related Proteins.

The two amino acid repeats, R2 and R3 of GAMyb, are compared with the repeats of other barley Myb homologs, Hv1, Hv5, and Hv33 (Marocco et al., 1989; Wissenbach et al., 1993), petunia Myb homolog, PHMYB3 (Avila et al., 1993), maize Myb homolog, ZmC1 (Paz-Ares et al., 1987), and human c-Myb (Majello et al., 1986). Shaded boxes indicate the amino acids that are identical to those of GAMyb.

GAmyb gene precedes that of the α-amylase gene expression in GA_3-treated aleurone layers. *GAmyb* mRNA levels rose dramatically within the first 3 hr after the addition of GA_3, whereas the maximum rate of increase in α-amylase mRNA levels occurred between 6 and 12 hr. Examination of earlier time points indicated that *GAmyb* mRNA levels began to increase within 1 hr in response to GA_3 (data not shown). Maximal *GAmyb* mRNA levels (6 to 12 hr) coincided with the maximal rate of accumulation of α-amylase mRNA, which is consistent with the possibility that GAMyb may regulate expression of the α-amylase genes.

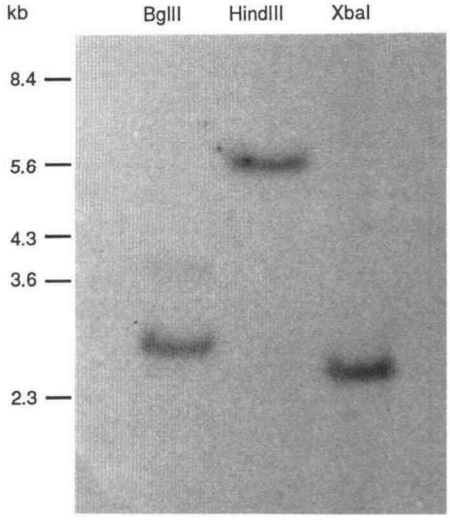

Figure 4. DNA Gel Blot Analysis of the *GAmyb* Gene in Barley Genomic DNA.

DNA (25 μg) was digested with BglII, HindIII, or XbaI. After blotting, the digested DNA was probed with a *GAmyb* gene–specific probe (a PCR product encoding 3′ sequences from 1430 to 2189). Positions of DNA molecular length markers are indicated at left in kilobases.

Cycloheximide Induces the Expression of *GAmyb*

To test whether *GAmyb* gene expression is also sensitive to the protein synthesis inhibitor cycloheximide, we performed RNA gel blot analysis using the same *GAmyb* probe and high-pI α-amylase cDNA probe. Figure 6 shows that the *GAmyb* mRNA levels in barley aleurone layers increased in response to either GA_3 or cycloheximide and that the increase was much greater in the cycloheximide-treated layers compared with GA_3-treated layers. When the tissue was incubated with both GA_3 and cycloheximide, the increase in *GAmyb* mRNA was greater than with either treatment. The effect of cycloheximide on *GAmyb* gene expression contrasts with that found for α-amylase gene expression. Cycloheximide did not induce α-amylase gene expression, and the GA_3-induced increase in α-amylase mRNA levels was inhibited by cycloheximide. This result agrees with an earlier report on the effects of cycloheximide on α-amylase gene expression (Muthukrishnan et al., 1983).

GAMyb Binds to the α-Amylase Promoter

To test whether GAMyb binds to the TAACAAA box in the high-pI α-amylase promoter, we expressed a glutathione

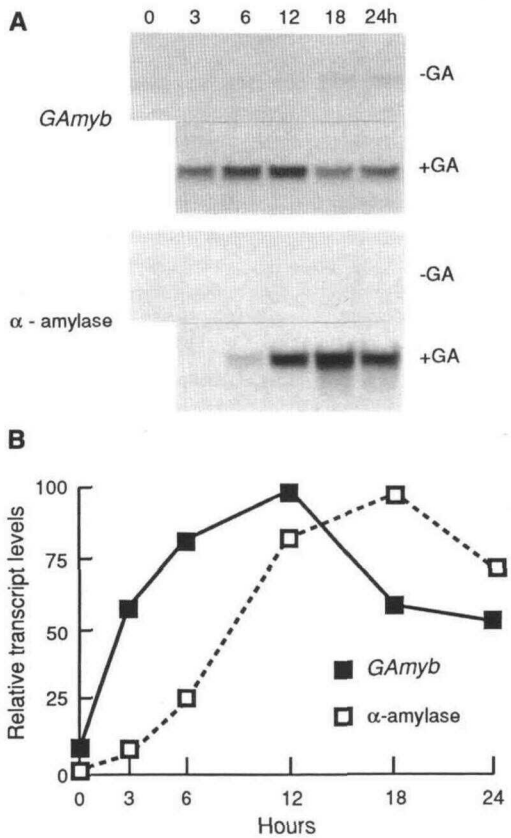

Figure 5. Effect of GA_3 on *GAmyb* and α-Amylase Gene Expression in Barley Aleurone Layers.

(A) RNA gel blot analysis of isolated barley aleurone layers that had been incubated with (+) or without (−) GA_3 and probed with a 3′ gene-specific *GAmyb* cDNA probe and a high-pI α-amylase cDNA. Numbers above each lane indicate hours (h) after the start of the treatment. **(B)** Quantitation of time course of *GAmyb* and α-amylase gene expression in GA_3-treated layers. mRNA levels were quantified by PhosphorImager and normalized to rRNA.

S-transferase (GST)–GAMyb fusion protein (recombinant GAMyb or rGAMyb) in *Escherichia coli*. Interaction between the affinity-purified rGAMyb and an oligonucleotide probe (w) containing an α-amylase promoter sequence (−149 to −128), including the TAACAAA box (Figure 7A), was assayed by gel mobility shift assays. Figure 7B shows that the rGAMyb fusion protein bound to probe w, resulting in the formation of a lower mobility complex (lane 3). In contrast, the GST protein alone failed to bind (lane 2). Competition with 100-fold molar excess of unlabeled w completely abolished complex formation between probe w and GAMyb fusion protein (Figure 8), indicating that binding is reversible.

To determine the nucleotides important for binding the *GAmyb* within the 22-bp w probe, we used a series of mutant probes containing single (m2 to m5) and multiple (m1) base pair mutations. Extensive mutation of the TAACAAA box to CTCGAGA (underlining indicates mutations) in probe m1 abolished binding of GAMyb (Figure 7B, lane 4), indicating that the GAMyb binding site includes at least part of the TAACAAA box. A similar mutation of the TAACAAA box in the α-amylase promoter had been shown previously to cause a decrease in GA-induced expression in transient expression analyses (Gubler and Jacobsen, 1992).

As shown in Figure 1, the TAACAAA box region contains two putative Myb binding sites that partly overlap: TAACAAAC and AAACTCCG. To determine whether both these sequences are GAMyb binding sites, probes containing single base pair mutations of the core Myb binding sequence, AAC, were tested for their ability to bind GAMyb. Mutation of the first A residue in the core sequence of Myb binding sites, AAC, has previously been shown to abolish binding of vertebrate Mybs (Weston, 1992; Ording et al., 1994). Figure 7B shows that GAMyb failed to bind m2 (lane 5), which contains a single base pair mutation (TGACAAAC) in the AAC core of the 5′ putative Myb binding site. In contrast, a similar mutation in the 3′ putative binding site (AGACTCCG) had no effect on GAMyb binding (lane 6). These results indicate that the 3′ site is not critical for binding of GAMyb. Additional single mutations of the TAACAAAC element were introduced to confirm the 3′ end of the binding site. GAMyb failed to bind to m4 and m5 (lanes 7 and 8, respectively), which carry single mutations at the 3′ end of the octameric binding site. Competition experiments were also performed with the mutant oligonucleotides (m1 to m5). Figure 8 shows that only m3 strongly competed for binding with the probe w, confirming that GAMyb can bind to the mutated sequence TAACAGAC, which resembles the TAACAAA box counterpart, in barley low-pI α-amylase promoters. Oligonucleotides m4 and m5 competed weakly for binding.

The TAACAAA box has been shown to be functionally important in transient expression assays (Skriver et al., 1991; Gubler and Jacobsen, 1992). The clustered point mutations introduced in m1 that abolished *GAmyb* binding have been shown previously to reduce greatly the GA responsiveness of the high-pI α-amylase promoter in transient expression experiments (Gubler and Jacobsen, 1992). To test further whether

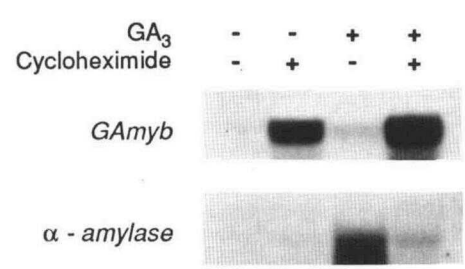

Figure 6. Differential Effect of Cycloheximide on *GAmyb* and α-Amylase Gene Expression.

RNA gel blot analysis of isolated aleurone layers treated with GA_3 and cycloheximide for 6 hr. The filters were probed with the same probes as described in Figure 5.

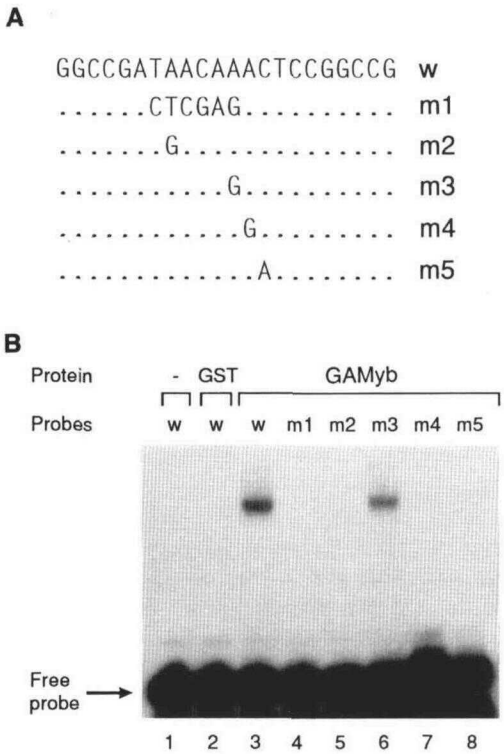

Figure 7. Binding of GAMyb to a 22-bp α-Amylase Promoter Fragment Containing the TAACAAA Box.

(A) Sequences of oligonucleotide probes used in gel mobility shift analyses. Probe w contains wild-type α-amylase promoter sequence from −149 to −128 (Jacobsen and Close, 1991). Probes m1 to m5 contain various mutations in the TAACAAAC box.

(B) Results of gel mobility shift analyses using the affinity-purified recombinant GAMyb protein and ^{32}P-labeled oligonucleotide probes shown in **(A)**. Lane 1 contains probe w alone; lane 2, probe w and GST; lane 3, probe w and rGAMyb; lane 4, probe m1 and rGAMyb; lane 5, probe m2 and rGAMyb; lane 6, probe m3 and rGAMyb; lane 7, probe m4 and rGAMyb; and lane 8, probe m5 and rGAMyb.

the GAMyb binding site is functionally important in conferring GA responsiveness to the α-amylase promoter, mutations used in the binding studies were introduced into the construct Am(−174)IGN shown previously to have all the sequences necessary for GA and abscisic acid control. The mutant promoter constructs were analyzed by transient expression in intact aleurone cells using microparticle bombardment. Figure 9 shows that introduction of a single base pair mutation, TGACAAAC, which abolished GAMyb binding (m2 in Figure 7B), strongly reduced GA-regulated expression, whereas the mutation TAACAGAC had no effect on expression or *GAmyb* binding. These results demonstrate that there is a close correlation between the *GAmyb* binding site and sequences that are functionally important in the GA response.

GAMyb Is a Transcriptional Activator of a High-pI α-Amylase Gene Promoter

To test the in vivo function of the protein encoded by the *GAmyb* cDNA, we determined whether GAMyb could transactivate a high-pI α-amylase gene promoter–β-glucuronidase (*GUS*) construct. Barley aleurone tissue was cobombarded with a *GUS* reporter gene fused to a high-pI α-amylase promoter, Am(−174)IGN (see Methods for full description) and a *GAmyb* effector plasmid (Figure 10A). The effector plasmid consisted of a rice *actin1* promoter fused to the *GAmyb* cDNA. Figure 10B shows that GUS activity from AM(−174)IGN, which is normally induced by GA_3, was also induced in the absence of GA_3 by the Act1.GAmyb effector construct. The increase in GUS activity in response to Act1.GAmyb expression was similar to that found with GA_3 alone. Expression of Act1.GAmyb did not increase GUS expression in GA_3 treatments, presumably

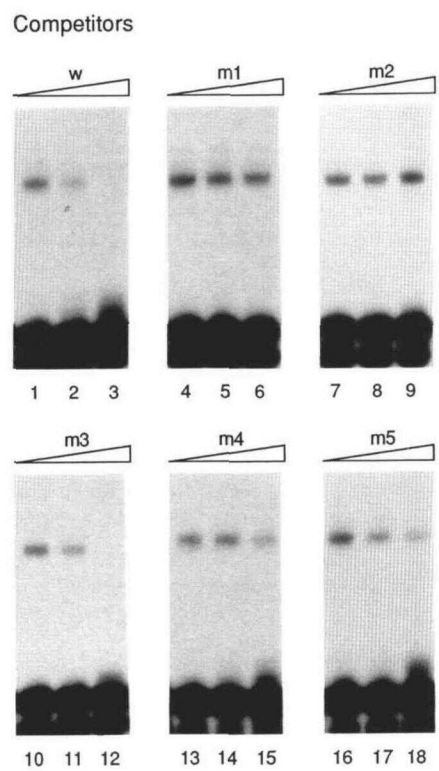

Figure 8. Competition for Binding of GAMyb to Probe w.

Unlabeled competitor oligonucleotides w (lanes 1 to 3), m1 (lanes 4 to 6), m2 (lanes 7 to 9), m3 (lanes 10 to 12), m4 (lanes 13 to 15), and m5 (lanes 16 to 18) were incubated with rGAMyb before the addition of labeled probe w. The left-hand lane of each gel (lanes 1, 4, 7, 10, 13, and 16) shows the protein–DNA complex formed with probe w in the absence of any competitor. Successive lanes show the effect of the addition of 10-fold (lanes 2, 5, 8, 11, 14, and 17) and 100-fold (lanes 3, 6, 9, 12, 15, and 18) molar excesses of competitor DNA.

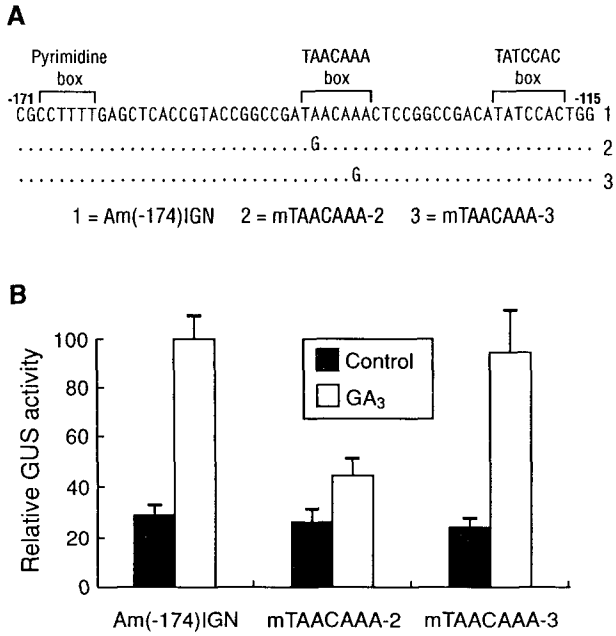

Figure 9. Transient Expression Analysis of High-pI α-Amylase Promoter with GAMyb Binding Site Mutations.

(A) Diagrams of wild-type and mutant α-amylase promoters. Am(−174)IGN contains a wild-type GAMyb binding site. mTAACAAA-2 and mTAACAAA-3 contain single base pair mutations introduced into the GAMyb binding site.

(B) GA_3 responsiveness of wild-type and mutant high-pI α-amylase promoters. Am(−174)IGN, mTAACAAA-2, and mTAACAAA-3 were bombarded into intact aleurone cells and incubated with no hormone (control) or GA_3. All GUS values have been shown relative to the activity of Am(−174)IGN. The error bars represent SE (n = 11).

because other transcription factors are limiting. Expression of a control gene (unrelated to *GAmyb*), Act1.bar, failed to transactivate Am(−174)IGN (data not shown). Mutation of the TAACAAA box (mTAACAAA-1) in the α-amylase promoter (as shown for m1 in Figure 7A) greatly reduced the response of the reporter construct both to GA_3 and also to the Act1.GAmyb effector construct. These results indicate that GAMyb is a transcriptional activator of the high-pI α-amylase promoter in barley aleurone cells. In addition, the results provide functional evidence that *GAmyb* activates expression via the TAACAAA box, a binding site for this transcription factor.

To test whether other plant Mybs can activate the high-pI α-amylase promoter, the maize *C1* cDNA was fused to the rice *actin1* promoter (Act1.C1; Figure 10A) and cobombarded with Am(−174)IGN. The maize *C1* cDNA failed to transactivate the reporter gene in the transient expression analysis. Furthermore, expression of the *C1* cDNA partially inhibited the GA_3 response. To test whether the *C1* cDNA was expressed in bombarded aleurone cells, cells were cobombarded with a *myc* cDNA (35SB-Peru) and Act1.C1. Only the cells with both

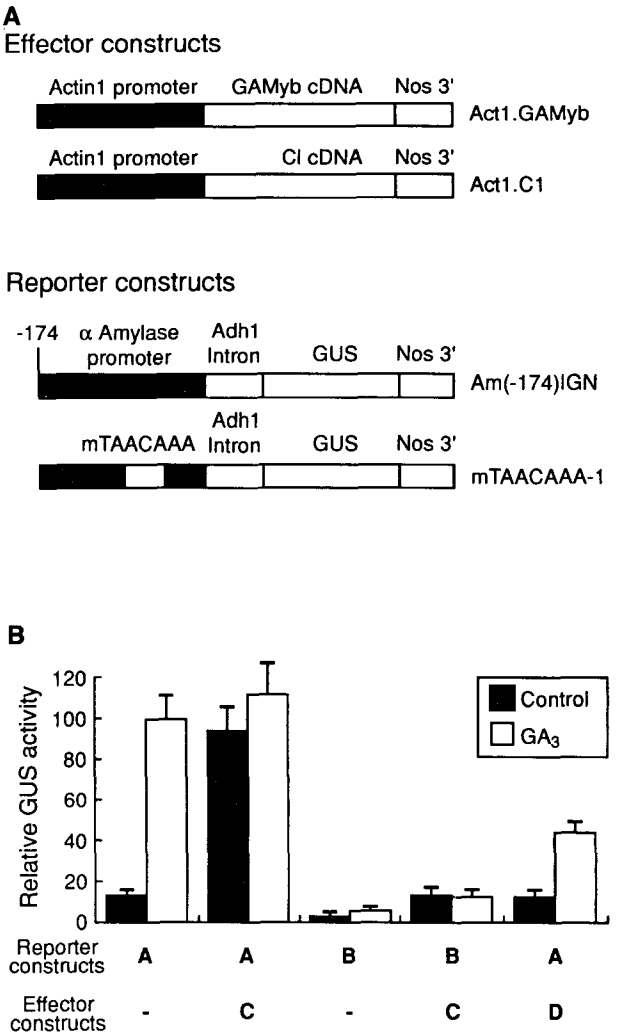

Figure 10. Transactivation of the High-pI α-Amylase Promoter by GAMyb in Barley Aleurone Cells.

(A) Effector and reporter constructs used in cobombardment experiments. The effector constructs contain the rice *Actin1* promoter fused to *GAmyb* cDNA (Act1.GAmyb) or maize *C1* cDNA (Act1.C1). The reporter construct Am(−174)IGN has the wild-type TAACAAA sequence, whereas mTAACAAA-1 has multiple point mutations in the TAACAAA box (CTCGAGA, shown as a white box in the promoter); these are the same as those in gel mobility shift probe m1 (see Figure 7).

(B) Response of high-pI α-amylase promoter to GAMyb and C1 expression in transient expression analyses. Intact aleurone cells were cobombarded with reporter constructs and effector constructs and incubated with no hormone (control) and GA_3. The error bars represent SE (n = 12 to 34).

plasmids produced red anthocyanin pigment (data not shown). It has shown previously that both classes of transcription factors are required for anthocyanin production in barley aleurone cells (Kalla et al., 1994).

DISCUSSION

There is now evidence that plant Myb-related proteins regulate a number of different aspects of plant morphogenesis. These include anthocyanin and phlobaphene biosynthesis (reviewed in Dooner et al., 1991), trichome differentiation (Oppenheimer et al., 1991), epidermal cell shape (Noda et al., 1994), and dehydration stress (Urao et al., 1993). We have now isolated and characterized a cDNA clone, *GAmyb*, that encodes a Myb-related protein that plays a crucial role in GA-regulated gene expression in barley aleurone layers. We postulate that this protein is part of the GA response pathway leading to α-amylase gene expression in aleurone cells.

GAmyb gene expression in barley aleurone layers was induced by GA. Comparison of the kinetics of expression shows that *GAmyb* expression is an early molecular event in response to GA and precedes α-amylase gene expression consistent with the possibility that the expression of the two genes may be causally linked. Our results provide three lines of evidence to support this hypothesis. First, we showed that GAMyb fusion protein binds to a GARC *cis*-acting element, the TAACAAA box, in a barley high-pI α-amylase gene promoter. By introducing mutations into the TAACAAA box and adjoining 3′ sequence, the binding site was shown to include the sequence TAACAAAC, which is very similar to c-Myb and v-Myb consensus binding site, YAACKGHH (Weston, 1992). Second, there is strong agreement between the GAMyb binding site as defined in vitro and the sequences that are functionally important in transient expression assays. We showed that multiple or single point mutations in the TAACAAAC binding site that abolish binding to GAMyb fusion protein also reduced the GA responsiveness of the high-pI α-amylase promoter. This provides strong evidence that the GAMyb protein functions in vivo to activate α-amylase gene expression through the TAACAAA box. Third, we demonstrated in transient expression experiments that GAMyb activates transcription of a high-pI α-amylase promoter fused to a *GUS* reporter gene in the absence of GA_3. Mutation of the TAACAAA box greatly reduced the ability of GAMyb to transactivate the α-amylase promoter construct, confirming that the TAACAAA box is the site of GAMyb binding and transactivation.

Figure 11 shows a model of the GA response pathway between the expression of the high-pI α-amylase gene and the initial GA signal based on our results. In this model, GA binds to a receptor, presumably on the plasma membrane, and activates a signal transduction pathway that triggers *GAmyb* gene expression. The newly synthesized GAMyb protein activates the expression of the high-pI α-amylase gene. In the absence of GA, we were able to activate the high-pI α-amylase promoter

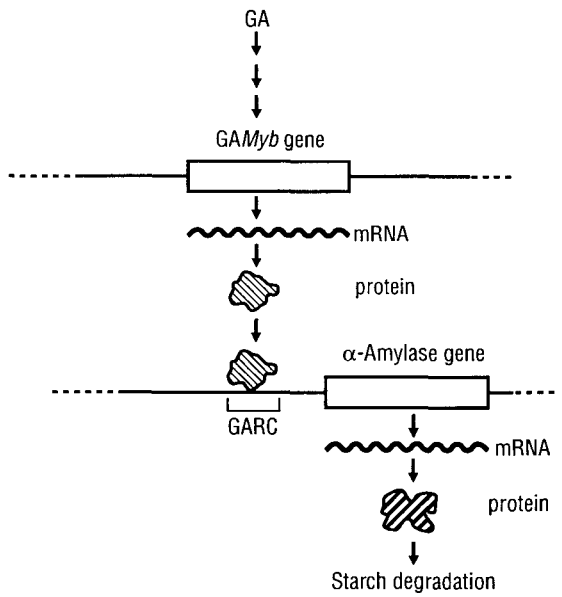

Figure 11. Proposed GA Response Pathway for High-pI α-Amylase Gene Expression in Barley Aleurone Cells.

activity by transiently expressing GAMyb under the control of a constitutive promoter. Our results indicate that the GAMyb can act as the sole GA-regulated transcription factor required for transcriptional activation of the high-pI α-amylase promoter. Other transcription factors necessary for high-pI α-amylase gene expression (e.g., factors that may bind to the pyrimidine and TATCCAC boxes) are likely to be present in non–GA-treated aleurone cells. These factors appear to be present in limiting amounts because overexpression of GAMyb in GA_3-treated aleurone cells did not result in higher expression of the reporter gene driven by the α-amylase promoter compared with non–GA_3-treated aleurone cells.

The wide occurrence of TAACAAA-like sequences in promoters of other cereal α-amylase genes (Huang et al., 1990) may indicate broad action of the GAMyb protein. Detection of a nuclear factor from barley aleurone layers, which binds to the TAACAGA box and associated 3′ sequences of a barley low-pI α-amylase promoter in a GA-dependent manner (Sutliff et al., 1993), is consistent with the model shown in Figure 11. It seems probable that the nuclear factor is GAMyb or a GAMyb complex because GAMyb was shown in this present study to bind to probe m3, which contains the sequence TAACAGA. Other GA-responsive gene promoters also contain sequences that resemble the GAMyb binding site in the barley high-pI α-amylase promoter. 5′ deletion analysis of the barley EII (1-3, 1-4)-β-glucanase gene promoter (Wolf, 1992) and wheat cathepsin B-like gene promoter (Cejudo et al., 1992) showed that the GA-responsive regions were downstream of −310 and −173, respectively. The sequence between −169 and −162 in the EII β-glucanase promoter, TAACAACC, is very similar to the GAMyb binding site, with only one base mismatch (Wolf, 1992).

The sequence between −140 and −135, GAACCGAA, in the cathepsin B–like gene promoter (Cejudo et al., 1992) may act as a binding site for a wheat GAMyb homolog.

Previous studies using the protein synthesis inhibitor cycloheximide have shown that the de novo synthesis of at least one intermediate factor is necessary to activate transcription of α-amylase genes (Muthukrishnan et al., 1983). Our results confirm this and indicate that the intermediate factor is GAMyb. In contrast to α-amylase gene expression, *GAmyb* gene expression is superinduced by either cycloheximide or cycloheximide and GA. These results are consistent with the idea that protein synthesis is not required for GA induction of *GAmyb* gene expression and that upstream signal pathway proteins must preexist. Therefore, it is reasonable to suppose that the *GAmyb* gene is the first GA-regulated gene in the pathway. In plants, a number of other hormonally regulated genes can also be induced by cycloheximide (Theologis et al., 1985; van der Zaal et al., 1987; Mundy and Chua, 1988; Franco et al., 1990; Shen et al., 1993; Yamaguchi-Shinozaki and Shinozaki, 1993).

The protein encoded by the *GAmyb* cDNA, like those of other Myb-related proteins, has structural characteristics that have been highly conserved during evolution and are utilized for DNA binding by many different transcriptional activators found in vertebrates, insects, slime mold, yeast, and plants (reviewed in Luscher and Eisenman, 1990). In animals and insects, the Myb domain includes three imperfect repeats, 51 to 53 amino acids in length. The GAMyb protein, like other plant Mybs, has a putative DNA binding domain that consists of two imperfect repeats corresponding to the second and third repeats of the Myb domain found in animal and insect Mybs (Paz-Ares et al., 1987; Marocco et al., 1989; Grotewold et al., 1991; Jackson et al., 1991; Oppenheimer et al., 1991; Shinozaki et al., 1992; Leech et al., 1993). The R2 and R3 repeats of c-Myb each contain three helices, and the third helix in each was found to be a recognition helix (Ogata et al., 1994). Nuclear magnetic resonance analysis of Myb interaction with DNA showed that the two recognition helices contact each other directly to bind to the specific base sequence AACNG. The R1 repeat is not essential for DNA binding (Luscher and Eisenman, 1990) but may play a role in stabilizing the complex (Ording et al., 1994). The GAMyb binding site, TAACAAAC, in the high-pI α-amylase promoter differs somewhat from the c-Myb and v-Myb consensus binding site, YAACKGHH, and the maize *P* Myb consensus binding site (CCT/AACC) (Grotewold et al., 1994). It is likely that these differences in binding specificities are due at least in part to amino acid substitutions in the R2 and R3 repeats in the Myb binding domain.

There is as yet no evidence of involvement of Myb proteins in other GA-regulated processes such as cell elongation or flowering, but the pigmentation of petunia corollas is regulated by gibberellic acid, and there is some indication that Mybs are involved. The expression of a number of genes encoding chalcone synthase, chalcone flavanone isomerase, and dehydroflavanol 4-reductase, which are coordinately expressed during petunia corolla development, has been shown to be induced by GA_3 (Weiss et al., 1990, 1992). One flower-specific petunia *myb* gene, *myb.Ph3*, which showed highest sequence identity with the barley *GAmyb* gene in the Myb domain, is predominantly expressed in petal epidermal cells of developing petunia flower buds (Avila et al., 1993; Solano et al., 1995). Binding site selection studies showed that Myb.Ph3 recognizes a consensus sequence TAACTAACAAAA (opposite strand shown; Solano et al., 1995). The petunia chalcone synthase *J* gene promoter, which directs expression of a reporter gene predominantly in epidermal cells of petunia flower buds, contains two putative MYB.Ph3 binding sites (Koes et al., 1990; Solano et al., 1995). MYB.Ph3 has been demonstrated to be a transcriptional activator of the chalcone synthase promoter in tobacco protoplasts. It thus seems possible that the MYB.Ph3 protein plays a similar regulatory role to that we have proposed for the *GAmyb* gene product. Experiments with cycloheximide indicate that protein synthesis is required for GA-induced chalcone synthase gene expression similar to that shown for α-amylase gene expression (Weiss et al., 1992).

Recent studies have shown that the *Atmyb2* gene of Arabidopsis (Urao et al., 1993) and the *C1* gene of maize (Hattori et al., 1992) are both induced by abscisic acid. The mutually antagonistic effects of these two hormones at the level of gene expression may involve, in part, the expression of mutually antagonistic Mybs that are under the control of abscisic acid or GA. Our results provide some support for this hypothesis. *C1* expression was shown to repress GA activation of *GUS* expression by the high-pI α-amylase promoter (Figure 10), presumably by competing with the GAMyb for the TAACAAAC binding site or by preventing GAMyb binding with other transcription factors that are required for transactivation of the α-amylase promoter.

In conclusion, this study opens up new avenues for studying the molecular mechanisms of GA action on α-amylase gene expression and other GA-responsive genes in barley aleurone. Studies under way to examine how GA initiates *GAmyb* gene expression hope to come closer to the initial events in the signal transduction pathway. We are also investigating whether GAMyb acts as a master regulator of genes, activating not only high-pI α-amylase genes but also other GA_3-regulated genes encoding low-pI α-amylases and other hydrolytic enzymes.

METHODS

Molecular Cloning of GAmyb cDNA

A barley (*Hordeum vulgare* cv Himalaya) cDNA library prepared from hydrated mature aleurone layers (Stratagene) was screened for putative *myb* cDNAs. The library was screened with a 174-bp polymerase chain reaction (PCR) product containing sequences that encode parts of the conserved R2/R3 domain of the maize *C1* cDNA (amino acids 49 to 106). The *C1* cDNA used for making the PCR product was a gift from K. Cone (University of Missouri–Columbia). The probes were labeled with ^{32}P-dCTP by random priming. Hybridization was in 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) and 0.1% SDS,

5 × Denhardt's solution (0.1% Ficoll, 0.1% PVP, 0.1% BSA), and 100 μg/mL salmon sperm DNA at 54°C with washing in 3 × SSC, 0.1% SDS at 54°C (Jackson et al., 1991). Positive plaques were purified by a further round of plaque hydridization. Excision of pBluescript SK− plasmid containing the *GAmyb* cDNA was performed according to the manufacturer's instructions (Stratagene). The cloned cDNA fragment was sequenced by the DyeDeoxy Terminator cycle sequencing method using an Applied Biosystems 370A DNA sequencer (Foster City, CA).

Rapid amplification of the 5′ end of cDNAs was performed by the 5′ RACE procedure as described by Frohman (1990). Poly(A)$^+$ RNA from gibberellin A_3 (GA_3)-treated barley aleurone layers was reverse transcribed using a gene-specific primer 5′-TGTTCTTCTGCACCGCG-TTC-3′. Following removal of excess primer, a 5′ poly(A) tail was synthesized on the single-stranded cDNA by using terminal deoxynucleotidyl transferase. PCR amplification was performed using a nested 3′ gene-specific primer 5′-GTGCTTCACGTACTCCAC-3′ and an oligo(dT)primer. PCR fragments were cloned by blunt end ligation into pCR-Script (Stratagene) according to the manufacturer's instructions. Positive clones were identified by colony hybridizations (Sambrook et al., 1989), plasmid DNA isolated, and sequenced.

Plasmid Construction

The construct Am(−174)IGN, which has been described previously (Jacobsen and Close, 1991), contains a fragment of a high-pI α-amylase gene promoter from barley (−174 to +54) fused to a reporter gene cassette (maize *Alcohol dehydrogenase1* intron 1/β-glucuronidase [*GUS*]/nopaline synthase 3′ terminator sequence). mTAACAAA-2 and mTAACAAA-3 constructs were created by using PCR to introduce single base pair mutations within the TAACAAA box of the Am(−174)IGN construct. Mutagenic forward primers (underlining indicates mutation) mTAA11 (5′-GCCTGCAGGTCGACTCTAGAGAATCGCCTTTTGAGCTCACCG-TACCGGCCGATGACAAACTCCGG-3′) and mTAA12 (5′-GCCTGCAGG-TCGACTCTAGAGAATCGCCTTTTGAGCTCACCGTAQCCGGCCG-ATAACAGACTCCGG-3′) and a reverse primer Am-41 (5′-GTGTGCTG-CGCAGCATGCCGG-3′) were synthesized. PCR was performed using the mutagenic forward primers and the reverse primer and Am(−174)IGN as template DNA. The PCR products were cut with PstI and cloned into the PstI site at −41 in Am(−41)IGN (Jacobsen and Close, 1991), thereby re-creating pAm(−174)IGN constructs with single base pair mutations at −142 (TGACAAA) and at −138 (TAACAGA).

The pGEX vector (Smith and Johnson, 1988) was used for production of glutathione *S*-transferase (GST) fusion proteins for gel mobility shift experiments. The pGEX.GAmyb construct was made by cloning a 1656-bp EcoRI fragment containing the *GAmyb* coding region (residues 1 to 352) into the pGEX-3X vector.

GAmyb and *C1* effector constructs used in transient overexpression experiments were synthesized by cloning fragments containing the *GAmyb* and *C1* coding regions into the multicloning site of the plant expression vector p113Act1.cas (Graham and Larkin, 1995). The p113Act1.cas vector contains the rice *Actin 1* promoter (McElroy et al., 1990) and 5′ untranslated leader sequence (including intron 1) fused to a multicloning site–nopaline synthase 3′ terminator. The Act1.GAmyb construct used in overexpression experiments was prepared by first cloning the EcoRI *GAmyb* fragment containing the coding region into the EcoRI site of the pBluescript SK− vector. The entire EcoRI insert was then cut out as a HindIII-XbaI fragment and cloned directly into the multicloning site of the p113Act1.cas. The Act1.C1 construct containing the entire coding region of the *C1* cDNA cloned downstream of the rice *Actin1* promoter was also prepared using a similar strategy. The Actin1.bar construct (pDM302) containing the phosphinothricin acetyl transferase gene cloned downstream of the *Actin1* promoter has been described previously (Cao et al., 1992). The 35.S.B-Peru construct was kindly donated by V. Chandler (University of Oregon, Eugene).

RNA Blot Analysis

Aleurone layers were prepared from grains of cultivar Himalaya (1985 harvest, Washington State University, Pullman) as described previously (Chrispeels and Varner, 1967). The isolated layers were isolated from half grains without embryos that had been imbibed for 3 days and incubated in flasks containing 2 mL of 10 mM $CaCl_2$, 150 μg mL^{-1} cefotaxime, 50 units mL^{-1} nystatin, and either no hormone (control) or 10^{-6} M GA_3 at 25°C for various times. Following hormone treatments, the layers were stored in liquid nitrogen until required. Aleurone layers that were to be treated with cycloheximide were preincubated with 50 μM cycloheximide, 10 mM $CaCl_2$, and antibiotics for 30 min before GA_3 was added.

RNA was isolated from aleurone layers, according to Chandler and Jacobsen (1991), with one minor modification. Bentonite was omitted from homogenization medium. For RNA gel blot analysis, 20 μg of aleurone RNA was fractionated in 1% agarose gel containing formaldehyde and blotted onto nylon membrane. The blots were hybridized with a ^{32}P-dCTP–labeled 991–bp PCR product containing *GAmyb sequences (nucleotide 1198 to 2189). The probe was shown to be gene* specific by genomic DNA gel blot analysis (data not shown). Hybridization was performed at 42°C in 6 × SSC, 5 × Denhardt's solution, 0.1% SDS, and 50% formamide. The blots were washed in 0.1% SSC, 0.1% SDS at 65°C. After autoradiography, RNA gel blots were stripped of the *GAmyb* probe and reprobed with a 1.1-kb DNA fragment containing the pHV19 cDNA clone, a barley high-pI α-amylase cDNA isolated from GA_3-treated aleurone layers (Chandler et al., 1984), and a 9-kb wheat rRNA clone, pTA71 (Gerlach and Bedbrook, 1979). All DNA probes were labeled by oligonucleotide priming (Feinberg and Vogelstein, 1983). mRNA transcripts were quantified using a Molecular Dynamics PhosphorImager and ImageQuant software (Sunnyvale, CA). *GAmyb* and high-pI α-amylase mRNA transcript levels were normalized to rRNA levels to overcome error in RNA quantitation by spectrophotometry.

DNA Isolation and Gel Blot Analysis

DNA was isolated from etiolated leaves from 6-day-old barley seedlings, using the method described by Dellaporta et al. (1983). For detection of the *GAmyb* gene in genomic DNA, 25 μg of genomic DNA digested with BglII, HindIII, or XbaI was fractionated on a 1% agarose gel and blotted onto a nylon membrane. The blot was hybridized with a ^{32}P-dCTP–labeled PCR product that contained sequences between nucleotides 1430 and 2189. Hybridization and washing conditions were exactly as described above for the RNA blots.

Production and Purification of GAMyb Fusion Protein

Purified proteins for gel mobility shift assays were prepared from XL1-Blue *Escherichia coli* cells transformed with the pGEX-GAmyb construct, essentially as described by Urao et al. (1993). The transformed cells were grown in 500 mL of 2 × YT medium containing 100 μg mL^{-1} ampicillin and 1 mM isopropyl β-D-thiogalactoside for 6 hr at 25°C to induce the production of GST fusion proteins. The cells were

harvested by centrifugation and resuspended in lysis buffer (10 mM Tris-HCl, pH 8.0, 0.4 M NaCl, 5 mM $MgCl_2$, 5% glycerol, 0.5 mM EDTA). The cells were centrifuged again, and the pellet was resuspended in lysis buffer. The cells were lysed by a cycle of freezing and thawing followed by four 10-sec bursts of sonication. Triton X-100 and phenylmethylsulfonyl fluoride were added to the lysed cells to the concentration of 1% and 100 μg mL^{-1}, respectively. The lysed preparation was centrifuged, and the supernatant was mixed with pre-swollen glutathione Sepharose-4B beads (Pharmacia, Sweden) and incubated at 20°C for 2 min. After absorption, the beads were spun down and washed three times with 50 mL of 75 mM Hepes-KOH, pH 7.9, and 150 mM NaCl. The fusion proteins were eluted by washing the beads in 200 μL of 75 mM Hepes-KOH, pH 7.9, 150 mM NaCl, and 5 mM reduced glutathione. Glycerol was added to the eluted protein to a final concentration of 10%, and aliquots were snap frozen in liquid nitrogen and stored at −80°C.

Gel Mobility Shift Assays

Complementary oligonucleotides with 5′ overhangs containing native and mutant sequences of the high-pI amylase promoter (−149 to −128) were synthesized and are as follows: w (only top strand is shown, and mutations are underlined), 5′-GGCCGATAACAAACTCCGG-3′; m1, 5′-GGCCGACTCGAGACTCCGG-3′; m2, 5′-GGCCGATGACAAACTCCGG-3′; m3, 5′-GGCCGATAACAGACTCCGG-3′; m4, 5′-GGCCGATAACAAGCTCCGG-3′; and m5, 5′-GGCCGATAACAAAATCCGG-3′. After annealing, the complementary oligonucleotides were 5′ end labeled using ^{32}P-dCTP and the Klenow fragment of DNA polymerase I and purified by electrophoresis on polyacrylamide gels. Oligonucleotide binding reactions with the bacterially produced GAMyb fusion protein were performed in 10 μL of binding buffer (25 mM Hepes-KOH, pH 7.9, 50 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 10% glycerol, and 0.5 μg mL^{-1} BSA) with 2 μg of poly(dI-dC)–poly(dI-dC), 150 ng of GAMyb fusion protein, and 0.9 ng of a ^{32}P-dCTP–labeled olgionucleotide probe (40,000 cpm). After incubation at 20°C for 10 min, the samples were run on a 6% polyacrylamide gel containing 5% glycerol in 0.25 × Tris-borate-EDTA buffer at 140 V. After electrophoresis, the gels were dried and autoradiographed.

Transient Expression Analysis

Himalaya barley half seeds were prepared for particle bombardment as described by Lanahan et al. (1992). Plasmid DNA purified on Qiagen tips (Diagen, Hilden, Germany) was coated onto 1.6-μm gold particles, essentially as described by Hunold et al. (1994). For expression analysis of mutant α-amylase promoter constructs, 0.5 μg Am(−174)IGN or mAm6-7 constructs were precipitated onto 0.75 mg of gold. For experiments involving GAMyb overexpression, 1.5 μg of effector constructs (pAct1.GAmyb or pAct1.C1) and 0.5 μg of reporter constructs (Am(−174)IGN or mTAACAAA) were precipitated onto 0.75 mg of gold. Each experiment was highly replicated ($n > 12$). Six barley half grains were bombarded using a helium particle inflow gun (Finer et al., 1992). After bombarding, the six half seeds were cut longitudinally along the groove, resulting in two equal quarter grains that were then distributed into flasks containing 10 mM $CaCl_2$ (control) or 10 mM $CaCl_2$ and 1 μM GA_3. Both incubation solutions contained cefotaxime and nystatin, at the same concentrations as described above. After incubation for 24 hr at 25°C, the grains were frozen and stored at −70°C. Preparation of extracts and assays of GUS activity has been described previously (Lanahan et al., 1992).

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